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Sequential Preconditioning and Its Effect on Crude Oil Bioremediation by Indigenous Soil Bacteria

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Abstract. Preconditioning has been proved to result in a significant increase in adaptability of biological organisms to adverse environmental stimulations and various stressful conditions. Accordingly, in a new approach attempt was made to enhance hydrocarbon degradation ability of selected potential isolates from a petroleum polluted soil through sequential adaptation. Firstly 8 isolates selected based on their growth at 5% (v/v) crude oil. Second selection performed based on their ability to adapt gradually them to 10%, 20% and 50% (v/v) crude oil concentration respectively, at simulated natural conditions (6.5 pH, 32°C). Isolates transferred to higher concentration of crude oil every 14 days. Their exponential growth rate served as an indicator of their adaptation success. Selected bacteria identified biochemically and morphologically according to Bergey’s manual accompanied by 16s rRNA sequencing. Bacterial species were identified as *P. putida*, *A. lwoffi*, *A. hydrophila*, *P. stutzeri*, and *A. johnsonii*. Bacterial isolates were tested for their biodegradation ability individually and as a consortium; based on gas chromatographic analysis the consortium proved to be more efficient in n-alkane degradation as 93% of C8-C33 was removed in liquid cultures and 70% of hydrocarbons in artificially polluted soil, in 28 days. In addition ratios of n17/pristane and n18/phytane degradation supported the promising bioremediation ability of the consortium. Comparison between adapted and un-adapted bacteria revealed that aliphatic degradation enhanced up to 40% in soil. In general the results suggest that preconditioning bacterial isolates to higher oil concentration can significantly enhance their biodegradation ability especially as a consortium.

Keywords: Bacterial biodegradation, Bacterial consortium, Crude oil bioremediation, Sequential adaptation, Soil pollution

Abbreviations:
v/v: Volume/Volume percent.; CFU: Colony Forming Unit; CFU/ml: Colony Forming Units per milliliter; OD600nm: Optical Density at 600 nm; OD: Optical Density

1. INTRODUCTION

Industrialization is accompanied by inevitable complications among which pollutions and global warming are of utmost importance (Feulner et al., 2013). Oil spills are an important part of pollution and can be very hard to clean up, requiring weeks to even years. Bioremediation is one of the best ways (Majone et al., 2014) to clean and remove oil spills. Bioremediation results in break down, consumption, and removal of hydrocarbons by biological organisms (Nikolopoulou, 2011, Othumpangat and Castranova, 2014, Prendergast and Gschwend, 2014).

Bacterial bioremediation has long been considered as one the most effective components of bioremediation and since native bacterial populations in any environment have a limited ability to consume and remove contaminants, attempts were made to enhance this trait. One possible and effective solution is to generate biotechnologically improved bacterial populations as a way to enhance degradation capacity; however, this method may cause yet other complications concerning introduction of genetically modified (GM) organism in the environment. The introduced GM bacteria may have an unknown adverse effect on the environment by influencing ecological factors or inflicting pathogenic effects on different members of the ecosystem (Megharaj et al., 2011). So, it looks as if necessary to develop an alternative way to enhance remediation capacities of native bacteria with the highest bioremediation range in terms of hydrocarbons and environmental stability for any ecosystem or climate that have the potential to be polluted by oil spills.

Preconditioning is a strategy that has been utilized in different fields of biology to enhance positive outcomes or prevent adverse results. Therefore, here bacterial populations were preconditioned by
subjecting them to a sequence of high crude oil concentrations in their medium (10, 20 and 50% v/v); the hypothesis was that exposing bacterial populations to higher crude oil concentrations will result in selection of individuals with better adaptation ability followed by increase in their number and consequent enhancement of bioremediation capabilities of the population. In doing so, effective measurements can be taken towards dealing with likely pollutions with the fewest number of complications. This work implements following objectives: 1) to find the most potential set of bacteria able to grow in high concentration of crude oil in laboratory, 2) to adapt them sequentially to higher concentrations of crude oil, and exploit their remediation capacity at their natural habitat pH and temperature, and 3) to compare bacterial degradation efficiency as individual strains and in consortium before and after adaptation in liquid culture medium as well as artificially polluted soil.

In this work, aliphatic fraction of crude oil used to monitor bacterial isolates performance due to their abundance in crude oil composition regardless of the crude oil origin (Maioli, 2011), to test the efficiency of degradation by adapted bacteria and also to compare oil removal efficiency between adapted and non-adapted groups (Mana Capelli et al., 2001).

2. MATERIALS AND METHODS

2.1 Sample collection

Soil samples collected through judgmental sampling at vicinity of a refinery plant in Port Dickson, Negeri Sembilan, Malaysia (2°31'43.2"N 101°49'14.6"E, within 100m radius). Selection of sampling sites performed by visual selection from high contamination to moderate contamination based on the proposed method by Zhang and colleagues (2007). In total twenty-seven samples of soil were collected from depths of 0–15 cm using an auger. The samples were put into sealed sterile glass containers, stored on ice, and transported to the laboratory for biological analysis.

We measured pH and temperature at the sampling locations. pH measured at depths of 10 and 20 cm and at the soil surface in 0.01 M CaCl2. A digital thermometer (11047, DeltaTrack®, USA) used to measure temperature. Temperature measured at different depths of 15, 30, and 45 cm and at the surface of soil. We measured the temperature over a three week period at time points of 8–11 am, 1 –4 pm, and 9–11 pm.

2.2. Isolating oil degraders

2.2.1. Bacterial enrichment

Crushed, mixed, and air dried soil samples sieved through a 2 mm sieve, 10 g of soil was added into Trypton Soy Broth (TSB) amended with 1% v/v crude oil. Sample incubated for one week in a shaker incubator (200 rpm) at 36°C and enriched every three days. Then isolates were transferred into Trypton Soy Agar (TSA) plates supplemented with 1% v/v crude oil via pour plate method (Pepper and Gerba, 2005; Reddy et al., 2007). They were kept for 7 days in an incubator at 36°C. Later colonies showing different morphological appearances were cultured in liquid Mineral Salt Medium containing 5% v/v crude oil; after 7 days those colonies that exhibited good-to-moderate growth were selected for further analysis; judgment was done based on amount of growth determined using McFarland’s standard. Crude oil was sterilized passing through a 0.2 µm syringe driven filters (Thermo Scientific, UAS).

2.3. Relation between colony counting units and optical density

We required constant monitoring of the bacterial counts at sequential adaptation process. So, we attempted to determine a relation between the Colony Count Unit (CFU) of each selected isolate and its optical density (OD). To do so, isolates cultured in Mineral Salt Medium (MSM) containing 5% v/v crude oil with a pH of 6.5, in a shaker (120 rpm) at 32°C for one week. Then, we assembled four serial dilutions in which the bacterial concentrations were 1, ½, ¼, and 1/8 of the original levels, prepared under the same conditions. Their OD600, and their CFU, determined for each of these inoculations over a range of concentrations. Colony counting units and optical density were related using a linear regression equation.

2.4. pH and temperature optimization

2.4.1. Onsite pH and temperature measurements

To determine optimum values for pH and temperature, the isolates cultured in MSM amended with 5% v/v crude oil with different pH values and were incubated at different temperatures. Values of pH varied as follows: 6, 6.5, 7, and 7.5, at this stage temperature was kept constant at 36°C. In order to find the optimum temperature, isolates kept at different temperatures of 28, 30, 32, 34, and 36°C, while the pH was 7. Experiment conducted in triplicate and the mean data comparison performed using one-way
ANOVA. For each set of pH and temperature optimization, two sets of controls prepared, one without inoculation and the other without adding crude oil; no bacterial growth was detected in either of them.

![Graph showing soil pH and temperature at different depths](image)

**Fig. 1:** a) Soil pH at different depths, b) soil temperature at different depths and time points

### 2.5. Bioremediation evaluation

#### 2.5.1. Sequential adaptation

1.6 × 10^7 cells ml⁻¹ of each isolate was transferred into MSM containing 10% v/v crude oil and kept in shaker incubation, 120 rpm, 36°C. After 14 days, isolates that could sustain and thrive were transferred at a concentration of 1.6 × 10^7 cells ml⁻¹ into fresh MSM supplemented with 20% v/v crude oil and incubated in a shaker incubator, at 120 rpm, 34°C, followed again after 14 days by transferring 1.6 × 10^7 cells ml⁻¹ cells from each flask to culture flasks containing 50% v/v crude oil (incubated and shaken at 32°C, 120 rpm). Temperature and pH kept at 7 at the first stage of adaptation later the temperature for 20%, and 50% v/v of crude oil decreased to 34°C, 32°C respectively. The pH kept at 6.5 when the crude oil concentration was 20% and 50% v/v. As OD values do not guarantee cell vitality in order to make sure that cultures are growing and viable, CFU counts performed for each isolate on 7th, 14th, 21st, and 28th day.

In order to determine the quality of growth and to determine if the bacteria are thriving growth curve analysis was performed and doubling times were calculated for each isolate and at each different concentration.

#### 2.5.2. Biodegradation ability of individual isolates and their mixture

MSM medium was prepared and supplemented with 50% v/v crude oil. This medium used for both the
individual and bacterial consortium. In total, $1.6 \times 10^7$ cells ml$^{-1}$ of selected bacterial strains after adaptation ($3.2 \times 10^6$ of each in isolate case of consortium) were added into the experimental culture media to evaluate consortium biodegradability. Two sets of controls were prepared for each experiment as mentioned before.

![Graph a) CFU of bacterial consortium at different temperatures, b) pH](image)

**Fig. 2:** CFU of bacterial consortium at different a) temperatures, b) pH

### 2.5.3. Biodegradation ability of bacterial consortium in soil

Un-polluted soil samples collected from the sampling site and air-dried for one week in a clean laboratory with good ventilation. Soil homogenized and passed through 2mm mesh and further dried in oven at 60°C for one hour. Prior to biodegradation study, three sets of 500g of soil samples autoclaved at 121°C for 15 min to remove any bacteria, if present. 20% v/w of crude oil, 100g each, added into both sets of soil samples; one un-inoculated set (control) and the second set using an inoculated soil sample with no crude oil.

### 2.5.4. Extraction of oil residue

An equal amount of n-hexane was added to the liquid medium after 28 days. The solution was shaken thoroughly. After letting the emulsion settle down the residues separated. In order to extract the oil residue from soil, 10 ml of n-hexane was added to the soil and shaken for five minutes. After settling down, the top mixture was separated. To completely separate the soil particles, the residue phase was mixed with n-hexane and centrifuged for 20 minutes at 3000 rpm.

### 2.5.5. Gas chromatography analysis

Mixture was passed through a 0.2 µl filter to separate the bacteria, if present, before being injected into a gas chromatogram. Split ratio was 1:20. The equipment used for gas chromatography was a GS-MS 5973, containing a 6890 GC and a 5973 MSD (Agilent technologies®, USA). Fused silica gel column used with dimensions of 30 m × 0.25 mm. Helium was the carrier gas. The initial temperature kept at 60°C and the final temperature reached to 290°C. Temperature increased 15°C every minute.
2.6. Identification of isolates

Biochemical and molecular tests used for identifying bacterial isolates (Vasanthakumari, 2009). They identified by their morphology as well, using Bergey’s manual. We used gram staining, oxidase, nitrate reduction, carbohydrate fermentation, and catalase tests. To perform molecular identification First 500 base pairs of the 16S rRNA gene amplified and then sequenced (first base laboratories, Malaysia). Sequencing results compared and aligned with the online NCBI, BLAST® database.

3. RESULTS AND DISCUSSIONS

3.1. Bacterial isolation

Eight colonies out of 12 total colonies obtained from enrichment method were able to show good and moderate growth in MSM supplemented with 5% (v/v) of crude oil. Line regression of growth of each bacteria identified as explained (result are not shown).

High numbers of oil degrading microorganisms in an environment implies that those organisms are the active degraders of oil hydrocarbons. However it is known that these active microorganisms have a limited capacity for hydrocarbon removal and they will try other sources of carbon and energy if available. Any changes in the bacterial environment can be somewhat a stress and consequently a limiting factor; therefore, adding bacteria to the culture medium amended with crude oil causes bacteria to be exposed to a stressful condition (Kessington et al., 2014).

3.2. Temperature and pH optimization

The soil pH at different depths from the surface, 10, and 20 cm of depth is shown in Figure 2A. The soil pH became more alkaline with increasing depth,
increasing from 5.5 at the surface to 6.5 at a depth of 20 cm.

Results of soil temperature measurements at sampling site are shown in Figure 2B. Soil temperature absorption and desorption used to select time points (Müller, 1998). Pattern of soil temperature at the surface was nearly the same at different time points. However, the graph shows that a gradual increase in soil temperature from morning until noon which then reached the highest temperature from 1–4 pm. At a depth of 15 cm the temperature fluctuations are more or less the same as surface fluctuations. But, temperature fluctuations are different at deeper depths. The mean average temperature was 32°C. Figure 3 shows results of optimum temperature and pH and their effect on the growth of bacteria.

Activity and growth of oil degrading bacteria also relies on the environmental conditions for example pH and temperature for instance, Plant residues at the surface could be considered as a reason for the acidic conditions of the soil surface (Xu et al., 2006). Increase in soil pH with increasing depth could be a result of salt wash off (Xu et al., 2006). Pollutions such as oil contamination and its natural bioremediation in the soil also have direct and indirect effect on the soil pH. The increase in soil pH with depth in our measurement is consistent with the results of other studies on tropical soil pH.

Although bacteria be active over wide range of temperature and pH but their optimal growth and activity happens in a limited range. Bacterial enzymatic activity depends on temperature and doubles with each 10°C rise in temperature. The ideal temperature is 15°C to 40°C and pH is 5.5 to 8 for microbial activity. The optimum range for hydrocarbon biodegradation in terms of temperature and pH is 20°C to 33°C and 6.5 to 8, respectively (Vidali, 2001).

It has been shown that environmental conditions have significant effect on the rate of biodegradation; in between, another study proved that the biodegradation increases at the optimum environmental conditions suitable for bacterial growth (Trupti and Dave, 2006).

3.3. Second bacterial screening

Figure 3 shows bacterial CFU counts at 10% and 20% (v/v) of crude oil. CFU count of bacteria showed that bacteria could grow well at 10% (v/v) of crude oil and this concentration was enough to support bacterial growth, hence no sudden decline in bacterial population; the toxicity level did not affect bacterial growth but, a remarkable change in growth of bacteria observed when concentration of crude oil increased to 20% (v/v). The longer exponential phase specifies that isolates were adapting themselves to the new conditions due to a significant increase in the number of colonies. CFU of isolates F, G, and H on day 28 decreased from 1.4E9, 7.0E8, and 1.2E9 to 0.1E8, 3.8E8, and 4.1E8, correspondingly. Their population size decreased further until their doubling time showed a negative value that indicates no growth at crude oil concentration of 50% (v/v). Doubling time of isolates decreases when the crude oil concentration increases from 10 to 20% (v/v); indicating that bacteria adapted well to the crude oil as they could grow faster in 20% (v/v) of crude oil than 10% (v/v). At 50% (v/v) of crude oil, doubling time of A, B, C, and D decreased more than their respective value at 10% (v/v).

Shaking the cultures has facilitated the exchange of gases from the solution to the surrounding as a result collision of substrates with bacterial enzymes increases and therefore removal happens at a higher speed (Andrew et al., 2014). Oil concentration is another important aspect influencing bacterial growth and production. The behavior of the bacteria in adaptation experiments proves the other researches that bacterial biomass changes as a response to the concentration of crude oil (Jinlan, 2012; Van Hamme et al., 2003). This justifies the more bacterial growth in lower concentration. It can be suggested that although bacterial isolates grow in much lower rate at lesser concentrations; but, they can still be active, which is a sign that adaptation to higher concentration of crude oil was successful. Toxicity level of crude oil at concentration of 50% (v/v) can be accounted as a reason for the failure of F, G, and H isolates as they might have been able to tolerate that toxicity. Because the solubility degree of hydrocarbons in the crude oil mixture may affect these bacterial isolates while the other were shown to have survived.

3.4. Identification of isolates

Five bacterial isolates identified biochemically and through 16rRNA sequencing as Gram-negative bacteria: Pseudomonas putida, Pseudomonas stutzeri, Acinetobacter johnsonii, Acinetobacter lwoffi, and Aeronomonas hydrophilla with the NCBI accession numbers of KT808260, KT808263, KT808264, KT808261, KT808262, respectively. Failed isolates were not identified by 16S RNA sequencing however they were identified biochemically; these three isolates were Gram-positive isolates, two were members of Bacillus sp. and one was a member of Rhodococcus sp. family.
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- **Pseudomonas putida**
- **Pseudomonas stutzeri**
- **Acinetobacter johnsonii**
- **Acinetobacter lwaffii**
After identification, the three failed isolates found to be Gram-positive bacteria. Their cell wall structure could be accounted for another failure reason as the toxic components of hydrocarbon accumulates in the cell wall membrane of bacteria and causes an increase in fluidity, which in turn causes swelling in the membrane and inhibits the normal functioning of the cell and its associated proteins and leads to eventual death. On the other hand the outer layer of the Gram-negative bacteria is highly impermeable and they are more tolerant to hydrocarbon toxicity (Eze et al., 2013).

### 3.5. Bioremediation analysis

Although each individual bacteria could degrade great quantity of aliphatic components of crude oil (67% in total); but, a considerable rise in the amount of aliphatic hydrocarbons removal (up to 93%) was detected when bacterial consortium added into the media amended with 50% (v/v) crude oil. Bacterial consortium could remove 92% of long-chained hydrocarbons (>C20) and 94% of the ones with carbon numbers less than 20. A similar trend was also seen for individual strains, individual strains removed 65% of hydrocarbons with carbon numbers more than 20 and 69% of short-chained hydrocarbons (Fig.4). Ratios of n-C17/Pr and n-C18/Ph in samples were shown to be less than the control. The ratio further increased for the consortium both in soil and medium. All these can lead to the conclusion that the consortium of bacteria was more efficient in removal of n-alkane in the crude oil sample.

As the selection of bacteria was based on their ability while they were in the consortium, their sensitivity to their surrounding further tested by growing them individually. As such, their hydrocarbon consumption efficiency tested on a mixture of hydrocarbon and not individual compounds. However, it was observed that although some isolates found to be from the same genus still their hydrocarbon biodegradation pattern was different. This might be due to physiology and differences in genetic characteristics of the strains in the same genera. Using n-C17/pr and n-C18/ph ratio as biodegradation marker confirmed that hydrocarbon degrading is performed by bacteria and has not been due to other factors (Wasify et al., 2014).
3.5.1. Bioremediation analysis in Soil

Analysis of crude oil residues after bacterial bioremediation showed that degradation of short chain \( n \)-alkanes was more than long chain \( n \)-alkane. 70% of the aliphatic components of crude oil removed of which 75% had less than 20 and 63% of which had carbon numbers more than 20 (Fig. 5).

However, the amount of biodegradation in soil is less than the liquid medium. The possible reasons for that could be the structure and texture of the soil that influence the availability and accessibility of contaminants for bacteria and the amount of available oxygen for their activity (Kuyukina et al., 2005). Yet, the amount of \( n \)-alkane removal during 28 days at concentration of 20% (w/w) is still high. Amphilic nature of surfactants lowers surface tension between different liquids or a solid surface and a liquid. Hence, make hydrocarbons accessible for bacteria.

3.5.2. Biodegradation ability of bacteria before and after adaptation

Calculating the percentage difference of \( n \)-alkane removal before and after the adaptation to high crude oil concentrations showed that adaptation seems to have enhanced the biodegradation ability of the selected strains up to 40%, especially in terms of long-chained alkanes (C\(_{20}\)-C\(_{33}\)) the ability of bacterial isolates enhanced up to 51%. Therefore, it can be concluded that the sequential adaptation of bacterial isolates increased the efficiency of hydrocarbon degradation (Fig. 6).

Prior exposure to petroleum contamination enhances the biodegradation ability of the organisms as those individuals with better adaptation or stronger enzymatic activity can be selected over the weaker individuals and also might provoke the microorganisms to express some of the silent genes in their genome that might result in better hydrocarbon removal. Other studies have also reported that exposing organisms to petroleum contaminants before
actual biodegradation studies has enhanced their metabolic activity (Rojo, 2009; Atlas and Philp, 2005). Genetic characteristics of each oil degrading organism play an important role in its ability to grow in different concentrations of crude oil (Ikuma, 2012). A clear drop observed in the amount of alkanes after introduction of strains that grew on petroleum amended medium for 24 days preceding their use for biodegradation of alkanes (Mana Capelli et al., 2001).

**Fig. 6:** Percentage difference removal of aliphatic components of crude oil by adapted and un-adapted bacterial isolates

To our knowledge, no reports on enhancing degradation ability of isolated bacteria by their sequential adaptation to higher concentration of crude oil exist and no existing report was found concerning on growing bacterial isolates at pH and temperature found at their natural habitats and measuring their biodegradation capability. We believe that combining these two approaches for choice of potential isolates has led to a more efficient degradation of petroleum hydrocarbons. The required time to degrade 93% and 70% of aliphatic compounds in the crude oil decreased in liquid sample and soil sample, respectively. As observed in another studies that just about the same degradation percentage achieved within 60-day period (Ghazali, 2004). Another study reported that degradation of 4.9% to 29.6% of crude oil occurred within 15 days (Okoh, 2003). In another report, around 60% of petroleum hydrocarbons degraded using a mixture of isolated oil degraders and nutrient supplements after 30 days (Roy, 2014) and yet in another study, hydrocarbon removal ability of isolated bacterial strains from oil polluted site was 83.49% (C8-C35) in a 75 days period when the crude oil concentration was 2% (v/v) (Varjani, 2015); while we succeed to have isolates degrade 93% of petroleum hydrocarbons through sequential adaptation and without adding any nutritional supplement or surfactant after 28 days.

4. **CONCLUSION**

Removal percentage difference before and after the adaptation showed that the adaptation seems to have enhanced the biodegradation ability of the selected strains up to 40%. In terms of long-chained alkanes (C20-C33) the ability of bacterial isolates enhanced up to 51%. Therefore, it can be concluded that sequential adaptation of bacterial isolates increased the efficiency of hydrocarbon degradation. Results of this study indicate that the consortium was capable of efficient removal of aliphatic hydrocarbons in just 28 days.

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